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Research Article

Anti-oxidant and Anti-inflammatory Activities of *Tetrastigma sulcatum* (Law.) Gamble Leaf Extract and its Fractions

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ABSTRACT
Hitherto unknown *in vitro* anti-oxidant and anti-inflammatory activities of crude ethanolic extract of leaf of *Tetrastigma sulcatum*, commonly known wild grapes, and its fractions were demonstrated. The total crude ethanolic extract as well as its hexane, toluene and ethyl acetate fractions were screened for antioxidant activity. The antioxidant activities were determined by DPPH, β-Carotene bleaching, reducing power assays. Total phenolic contents (TPC) of extracts and fractions ranged from 10 to 60 mg of GAE/g dry weight and correlated well with the activities observed. Screenings of the samples using TPA induced mouse ear oedema model indicated the anti-inflammatory activity of the total ethanolic extract. Interestingly the toluene fraction showed a better activity while activity of the hexane fraction was slightly inferior. The ethyl acetate fraction and the residue left after fractionation did not show anti-inflammatory properties. Details of the screening are reported.

keywords: *Tetrastigma sulcatum* leaf extract, fractions, anti-oxidant, local anti-inflammatory activity.

INTRODUCTION

The genus *Tetrastigma* (Vitaceae) is related to wild species of grapes. Many of the plants belonging to this genus have interesting biological activities. Majority of the reports available in the literature describe the activity of *T. hemsleyanum*. Liu *et. al.* reported antiviral activity of *T. hemsleyanum*[1]. Anti-inflammatory activity of the root of plant was subsequently reported[2-4]. Report about the anticancer activity of flavone isolated from *T. hemsleyanum* indicates its potential in cancer management[5]. Ethyl acetate extract of this species was shown to inhibit athymic mouse transplantation tumour by human hepatocarcinoma cell HepG-2[6]. Yong *et. al* reported phenolic profile of *T. hemsleyanum* leaf extract and correlated it with anti-oxidant and anti-proliferative activities[7]. Interestingly *T. hemsleyani* had obvious effect in inhibiting the proliferation and inducing apoptosis of human lung carcinoma H1299 cells[8]. Ethyl acetate fraction of root extracts from *T. hemsleyanum* possessed immunoregulatory effects[9]. Formulations prepared from mixture of the *T. hemsleyanum* with a few other plants along with propolis and bee venom was recently shown to be an effective composition for treating the rheumatism in Chinese medicine[10]. Scattered reports are available on the bioactivity of a few other species of the genus *Tetrastigma*. Antioxidant activities of *T. planicaule* stem extract[11], the leaves extract of tropical medicinal plant species Lipoi from Sabah[12] and leaves and stem extract of *T. thomsonianum*[13] are also reported. Flavonoids from *T. obtectum*, yet another species of the genus *Tetrastigma*, was shown to enhance glucose consumption in insulin resistance HepG2 cells[14]. It is also noteworthy that most of *Tetrastigma* species have better tolerance to the diseases and pests[15-18].

Tetrastigma sulcatum, commonly known as wild grapes, occurs abundantly in India. It is a large climber with thick stems and large green berry. Leaves are fleshy and ceriaceous, simple or forked with opposed simple tendrils without adhesive discs. The plant is abundantly found in Western Ghats from Malabar and W. Nilgiris to the Anamalais and Travancore hills up to 4500 ft. W. Peninsula. The extract was found useful in controlling number of fungal diseases of cultivated plants[19]. Preliminary phytochemical studies revealed the presence of polyphenols, flavonoids, leucoanthocyanins, catechol tannins, syringing glycosides, tannins, raphides and mucilage substances in the leaves of this plant[20]. Literature survey further indicates that anti-oxidant and anti-inflammatory activities of the leaf extract of the plant are not yet reported. We now report the anti-oxidant and anti-inflammatory properties of *T. sulcatum* leaf extract and its fractions.

MATERIALS AND METHODS

Authentication of plant and *Collection of leaves*

The plant *Tetrastigma sulcatum* from the experimental farm of Agharkar Research Institute at village Hol, District Pune, Maharashtra state, has been authenticated by Botanical survey of India, Pune. A specimen has been deposited at the herbarium of Botanical Survey of India (BSI), Pune (Voucher specimen number 189329). Leaves (3 kg) were collected for study.

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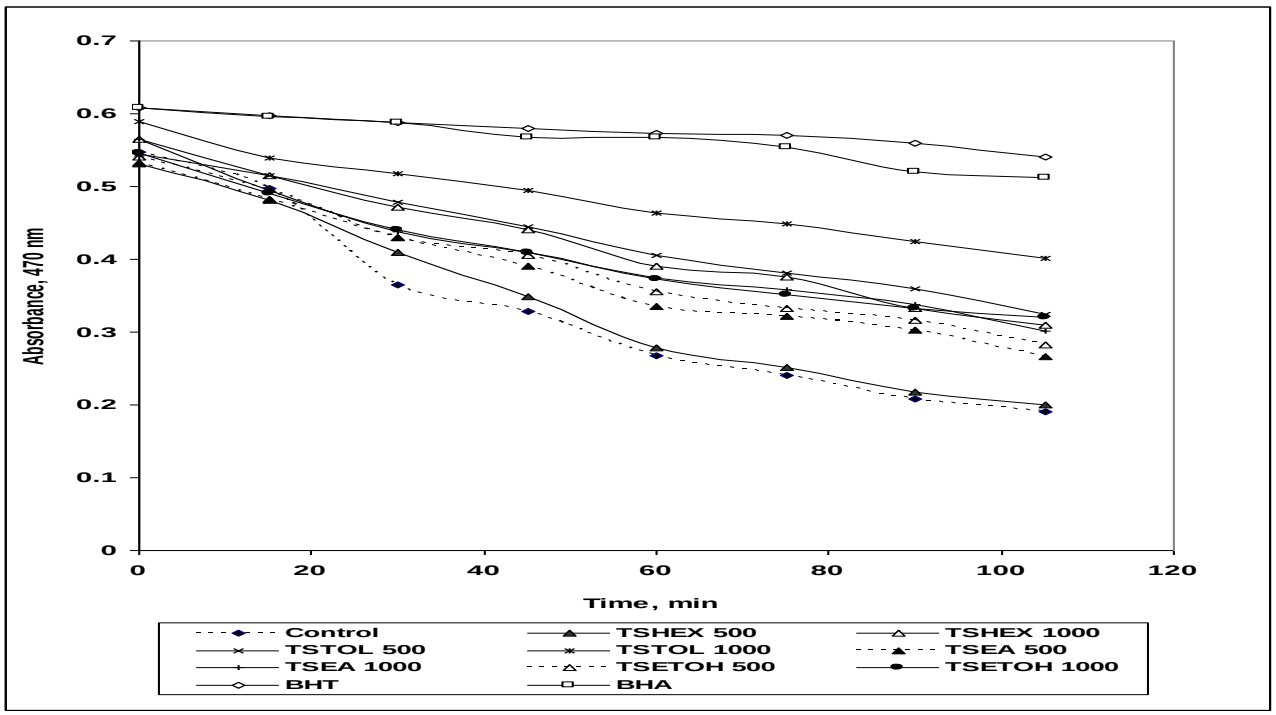

Figure 1: β-Carotene bleaching assay

Chemicals

Butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT) and tween-20 were purchased from Loba Chemicals, linoleic acid was purchased from SRL, β-carotene from HIMEDIA and Folin-Ciocalteu reagent was purchased from Qualigens. 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), ammonium per sulphate and indomethacin were purchased from Fluka, USA. 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was purchased from Sigma, USA. All the solvents used were of analytical grade.

Extraction

A part of the collected leaves (about 2.5 Kg) were separated, washed with water and shade dried. The dried leaves were powdered using a blender (1.2 Kg).

The powdered leaves (250 g) were packed in thimble of a Soxhlet apparatus and were extracted with ethanol (1.5 L) till the fresh extract from siphon was colourless (5 h). The alcoholic solution was evaporated under reduced pressure (about 20 mm of Hg) using a rotary evaporator to yield the total crude ethanolic extract. The procedure was repeated twice using the same quantity of powdered leaves to confirm reproducibility.

Fractionation of the total crude ethanolic extract

The total crude ethanolic extract was fractionated in the solvents of increasing polarity in the order hexane, toluene and ethyl acetate as follows:

The total crude extract (46 g) was suspended in hexane (3 x 200 ml) and the mixture was stirred for 30 minutes on a magnetic stirrer at room temperature. The combined hexane layer (560 ml) was evaporated under reduced pressure (about 20 mm of Hg) on a rotary evaporator yielding hexane soluble fraction, 'TSHEX'. The hexane insoluble portion of the crude extract (28 g) was suspended in toluene (3 x 200 ml). The mixture was stirred for 30 min at room temperature. The combined toluene layer (560 ml) was evaporated under reduced pressure on a rotary evaporator (about 20 mm of Hg) yielding toluene soluble fraction, 'TSTOL'. The part of residue (13.24 g) which was insoluble in hexane and toluene was suspended in ethyl acetate (3 x 200 ml). The mixture was stirred for 30 min. The combined ethyl acetate layer (570 ml) was evaporated under reduced pressure on a rotary evaporator yielding ethyl acetate soluble fraction 'TSEA'.

The portion of the crude extract that did not dissolve in hexane, toluene and ethyl acetate was collected separately. It was referred to as 'TSRES'.

Total crude alcoholic extract and the fractions obtained were screened to determine their anti-oxidant and anti-inflammatory activities.

Determination of Antioxidant activity

Antioxidant potentials of the total crude ethanolic extract and its hexane, toluene and ethyl acetate fractions were determined using *in vitro* DPPH, β-carotene bleaching and reducing power assays. The brief procedures for these assays are as follows:

DPPH Radical Scavenging Assay:

The standard protocol of DPPH assay[21] was followed with slight modifications. Solutions of different concentrations of extracts and standard, BHT in methanol (5, 10, 20, 30 and 50 µg/mL) were prepared. To each of the test solution (1 mL), DPPH solution in methanol (0.1 mM, 1 mL) was added. The total volume was made 4 mL using methanol. After 30 min incubation in the dark, absorbance was recorded at 515 nm. The percentage inhibition was calculated by using the formula:

$$\% \text{ Inhibition} = \frac{[A_0-(A_t- A_b)]}{A_0} \times 100$$

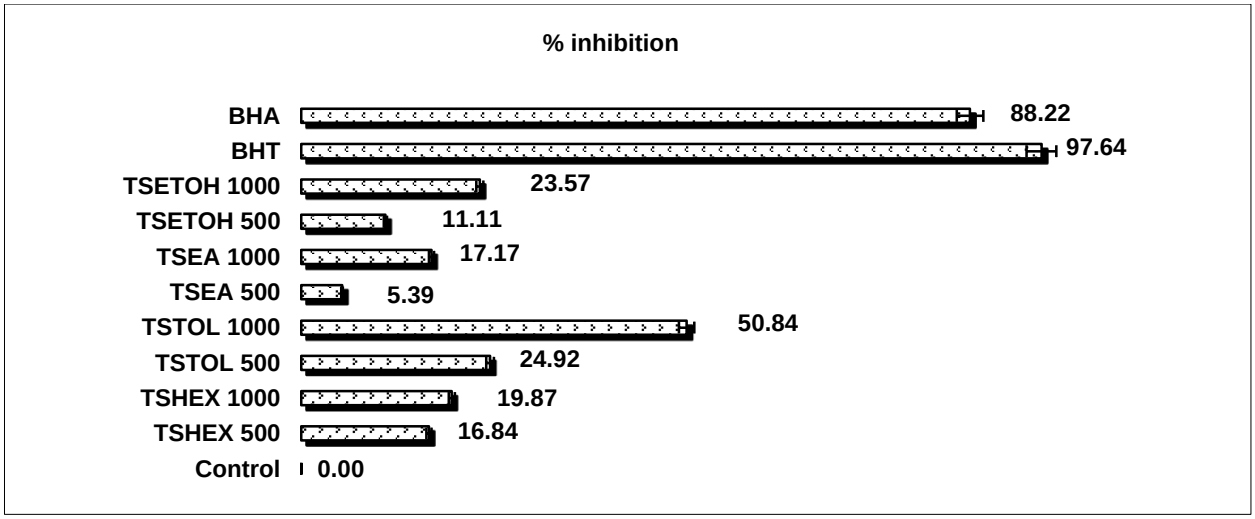

Figure 2: β-Carotene bleaching activity of *T. sulcatum* leaf extracts and fraction

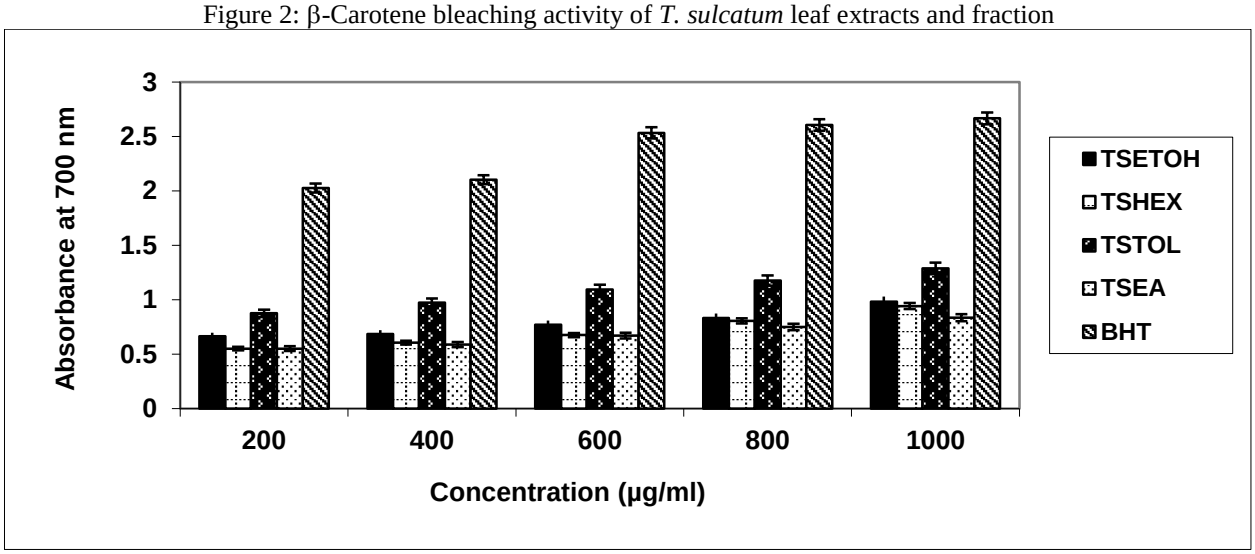

Figure 3: Reducing power assay

where, A_0 is the absorbance of control, A_t is the absorbance of test solutions/standard, A_b is the absorbance of blank solution. The per cent inhibition was plotted against the concentration of the test sample. The concentration of extracts which inhibits 50 % of DPPH radicals, referred as IC_{50} value, was determined from this graph. All the experiments were performed in triplicate.

β-Carotene Bleaching Assay:

Antioxidant activity was measured by the standard β-carotene -linoleic acid assay[22] with slight modifications. To the chloroform solution of β-carotene (3.34 mg in 1 mL), linoleic acid (40 mg) and Tween-20 (400 mg) were added. Chloroform was then removed at 40°C under vacuum (about 20 mm of Hg) using a rotatory evaporator. The resulting mixture was diluted with distilled water (10 mL). The volume of resulting emulsion was further made up to 100 mL with hydrogen peroxide (0.01 M). The test solutions of increasing concentrations (0.5 mg/mL and 1 mg/mL) of each sample and the standard solutions of BHA and BHT (1 mg/mL) in methanol were prepared. Aliquots (2 mL) of the β-carotene - linoleic acid emulsion was transferred into test tubes containing test samples (0.1 mL) and standards (0.1 mL) in methanol. A solution containing methanol (0.2 mL) and β-carotene- linoleic acid emulsion (2 mL) was used as a control. The blank was prepared in the same manner as that of test solution without β-carotene- linoleic acid emulsion. The test tubes were placed in water bath maintained at 40°C. Absorbance of all the samples at 470 nm was measured at zero time and after every 15 min till the colour of β-carotene disappeared in the control. All the samples were analysed in triplicate. The % inhibition was determined by using the formula:

$$\% \text{ Inhibition} = \frac{(A_{A(105)} - A_{C(105)})}{(A_{C(0)} - A_{C(105)})} \times 100$$

where, $A_{A(105)}$ is the absorbance of antioxidants at 105 min, $A_{C(105)}$ is the absorbance of control at 105 min, $A_{c(0)}$ is the absorbance of control at 0 min.

Reducing power assay:

The assay was carried out by standard procedure[23]. Solutions of the test samples (200, 400, 600, 800 and 1000 µg/ml each in methanol) were mixed with phosphate buffer (0.2 M, pH 6.6, 1 mL) and potassium ferricyanide (0.1 %, 1 mL). The mixture was incubated at 50^0C for 30

Table 1: IC_{50} values (DPPH assay) of *T. sulcatum* leaf extracts/fraction and their total phenolic contents

Sr. No.	Name of the Extract/Fraction/Std.	Total Phenolic content (mg/GAE)	DPPH Assay IC_{50} (µg/mL)
1.	Ethanolic Extract (TSETOH)	45.72 ±0.28	105 ±0.32
2.	Hexane soluble (TSHEX)	16.39 ±0.45	179 ±0.19
3.	Toluene soluble (TSTOL)	59.90 ±0.47	70 ±0.31
4.	Ethyl acetate soluble (TSEA)	10.20 ±0.34	190 ±0.42
6.	BHT	-	20 ±0.13

Results are expressed as mean ± standard deviation of three replicates.

Table 2: Effect of extract and fractions of *T. sulcatum* on TPA induced local inflammation in Swiss Albino mice.

Sr. No.	Treatment		Difference in ear thickness $\times 10^{-3}$ mm ±SEM	% inhibition of inflammation
1	Control		21.33±1.05	-
2	Crude extract (TSETOH)	0.5 mg/ear	16.50±1.43*	23.82
3		1 mg/ear	13.16±4.99*	39.24
4	Hexane fraction (TSHEX)	0.5 mg/ear	20.83 ± 1.10^{NS}	3.83
5		1 mg/ear	14.00±1.15*	35.36
6	Toluene fraction (TSTOL)	0.5 mg/ear	15.83±1.42*	26.92
7		1 mg/ear	11.66±1.35*	46.17
8	EtOAc fraction(TSEA)	0.5 mg/ear	22.83 ± 1.10^{NS}	6.57
9		1 mg/ear	19.16 ± 5.57^{NS}	11.54
10	Residue (TSRES)	0.5 mg/ear	19.50 ± 1.62^{NS}	8.45
11		1 mg/ear	19.83 ± 1.01^{NS}	9.97
12	Indomethacin	0.5 mg/ear	11.16±4.65*	48.48

Significant as compared to control $P< 0.05$

min. Aqueous trichloroacetic acid (10%, 1 mL) was added to the mixture and then centrifuged at 3000 rpm for 10 min. To the supernatant solution ferric chloride (1%, 2.5 mL) was added and absorbance was measured at 700 nm. BHT was used as the standard. All experiments were performed in triplicate. Reducing power was determined from the plot of absorbance versus the concentration of the test sample(s).

Determination of Total Phenolic Content

The total phenolic content was determined by the reported method using Folin-Ciocalteu reagent[24]. To a solution of the sample in methanol (100 µg/mL) Folin-Ciocalteu reagent (1mL) was added, the reaction mixture was allowed to stand for 5 minutes at room temperature and aqueous sodium carbonate (10 mL, 7%), was added to it. The reaction mixture was diluted to 25 mL with distilled water and kept at room temperature for 90 minutes. Absorbance of the resulting solution was measured at 750 nm. Total phenolic content of sample was determined using a calibration curve generated using absorbance of the standard solutions of gallic acid and is expressed as mg gallic acid equivalent (GAE)/g of sample. The samples were analysed in triplicate.

Determination of Anti-inflammatory Activity by Animal Experiments

Animal experiments were conducted as per the guideline suggested by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) under the Ministry of Statistics and Programme Implementation, Government of India. Approval of Institutional Animal Ethical Committee of Agharkar Research Institute was obtained to perform the proposed experiments.

Male Swiss Albino mice weighing 20 -25 g were obtained from the animal colony of Agharkar Research Institute (ARI). They were housed in polypropylene cages in an air-conditioned area at $25 \pm 2^{O}C$ with 10 to 14 h light and dark cycle and were maintained on Amrut brand balanced animal feed and water *ad-libitum*. Initially pilot experiments were carried out using three mice to decide dose for further study and six mice were used for individual treatments.

TPA induced local oedema in mice

Each of the samples was dissolved in 2 ml ethanol. From this stock solution standard solutions were prepared by dilution with ethanol.

Ear oedema was induced[25] on the right ear by the topical application of TPA (2.5 µg in 20 µl of acetone). The left ear (control) received the vehicle (acetone). The sample TSETOH, TSHEX, TSTOL, TSEA and TSRES were applied at 0.50 and 1 mg /ear, respectively, immediately after application of TPA. Indomethacin in a dose 0.5 mg / ear was used as a positive control. The thickness of ears was measured before and at 4h after application of TPA using a micrometer (Digitrix mark II, Japan). A group of six animals was used for each individual treatment.

The oedema was expressed as a mean ± SEM from these values and these results were analysed by Student's t test. Based on this the percentage inhibition of inflammation was calculated to assess the anti-inflammatory activity.

Statistical Analyses

The statistical analyses were performed using MS office-Excel 2003 for anti-oxidant activity. Values are presented as a mean ± standard deviation. Results were analysed by

Student's t test to assess significance of the anti-inflammatory activity.

RESULTS AND DISCUSSION

Extraction & Fractionation of the Total Crude Extract

Soxhlet extraction of *T. sulcatum* leaves in all the three experiments yielded approximately the same quantity of extract. Overall the total leaves extracted (750 g) yielded total crude ethanolic extract as a viscous, dark coloured semisolid having a peculiar odour (56.9 g, 7.59%). A part of it (46 g) on fractionation yielded hexane soluble fraction (TSHEX, 18.3g, 39.8%), toluene soluble fraction (TSTOL, 14.7 g, 32%), ethyl acetate soluble fraction (TSEA, 4.3 g, 9.3%) leaving the insoluble residue (TSRES, 8.7 g, 18.9 %).

Determination of Antioxidant Activity

Results of the determination of the anti-oxidant activity of total ethanolic extract of *T. sulcatum* leaf and its fractions are as follows:

DPPH Radical Scavenging Assay

Relatively stable DPPH radical has been widely employed to test the ability of compounds to act as free radical scavengers or hydrogen donors. The determination of IC^{50} values of *T. sulcatum* leaf extract, fractions and BHT by DPPH radical scavenging assay was done using the plot of % inhibition of DPPH free radical *versus* the concentration of the test solutions of the *T. sulcatum* leaf extract and its fractions. The values obtained are shown in Table 1. Lower IC^{50} value reflects higher antioxidant activity of plant extracts. It can be seen that IC_{50} inhibition for TSTOL was found to be 70 µg/mL while the IC_{50} values of the total crude ethanolic extract and other fractions were on the higher side. This indicates better activity of the toluene fraction of leaf ethanolic extract than that of the total ethanolic extracts or other fractions.

β-Carotene Bleaching Assay

In the β-Carotene bleaching assay, quantification of the decrease in the yellow color of β-carotene due to its reaction with radicals that are generated by oxidation of linoleic acid in the emulsion is made. This property is used in the evaluation of antioxidant activity of *T. sulcatum* leaf extract and its fractions. The activity is compared with the standard antioxidants BHA and BHT. Results of this assay carried out using crude ethanolic extract of *T. sulcatum* leaf and its fractions are indicated graphically as the plot of absorbance of the reaction mixture at 470 nm *versus* time (Fig. 1). It can be observed that the control showed a decrease in the absorbance with time due to the formation of peroxides in the absence of antioxidants. The standard and test solutions exhibited a slight decrease in absorbance, indicating inhibition of peroxide formation.

The per cent inhibition for different concentrations of *T. sulcatum* leaf extract and its fractions in comparison with that of BHA and BHT was determined. The per cent inhibition was calculated after 105 min. A plot of per cent inhibition thus determined against β-carotene bleaching activity of *T. sulcatum* leaf extract, its fractions, standard BHA and BHT against the concentration of the test samples is shown in Figure 2. It is seen from Fig. 2 that the extract as well as its other fractions found to reduce the rate of degradation of β-carotene in comparison with the control. The activity was found to increase with the concentration of *T. sulcatum* leaf extract and its fractions in the reaction mixture. In case of TSTOL the inhibition of peroxide formation was found to be 24.92 ± 0.28% at 500 mg/mL while the inhibition was found to be 50.84 ± 0.15% at 1000 mg/mL. Similar pattern is seen for the plots of other samples. This shows that the inhibition increased with concentration.

Reducing Power Assay:

The antioxidant activities of plant extracts have been correlated with their reducing powers. This assay is based on the principle that the substances which have reduction potential react with potassium ferricyanide (Fe3+) to form potassium ferrocyanide (Fe2+), which then react with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm. Higher the absorbance, higher is the reducing capacity of the samples. Figure 3 represents the reducing power of test samples in the form of a plot of absorbance at 700 nm *versus* the concentration of total ethanolic extract, its fractions and the standard antioxidant BHT. It can be seen that *T. sulcatum* leaf extract and its fractions exhibit reducing capacity at all concentrations. BHT shows higher absorbance than samples at all concentrations. The activity is found to be dose dependent for the samples.

Determination of Total Phenolic Content

Phenolic compounds are known as powerful chain breaking antioxidants. Phenols are very important plant metabolite because of their radical scavenging ability due to their hydroxyl groups. The phenolic compounds may contribute directly to antioxidant action[13]. It is, thus, well known that the phenolic compounds are responsible for antioxidant properties of the formulations. The phenolic content was, therefore determined by Folin Ciocalteu reagent. The total phenolic content, as gallic acid equivalent, of the total ethanolic extract and its fractions are reported in Table 1.

It was found to be highest in toluene fraction TSTOL (59.9 mg/g) followed by ethanolic extract TSETOH (45.72 mg/g), hexane TSHEX (16.39 mg/g) ethyl acetate TSEA (10.20 mg/g) fractions. The presence of phenolic in these extracts of *T. sulcatum* may be responsible for the antioxidant activity.

Determination of Anti-inflammatory Activity

Results of screening of the total crude ethanolic extract of *T. sulcatum* and its fraction by TPA induced local inflammation in Swiss Albino mice are shown in Table 2.

It is evident from the Table that crude ethanolic extract exhibited anti-inflammatory activity in a dose-dependent manner. It further shows that the toluene fraction was most active.

Oral administration of the crude ethanolic extract of *T. sulcatum* did not show any behavioural changes or toxicity up to 14 days in Albino mice. However on i.p. administration, there was a reduction in motor activity and ptosis up to 4 h indicating degradation or non-absorption by the oral route.

The results show dose-dependent and significant local anti-inflammatory activity of the extract and the hexane as

well as toluene fractions. The activity of the ethyl acetate soluble part and the insoluble residue of the extract were insignificant.

It would be further seen from the Table that the activity is better at the higher concentration. However the activity of the crude extract and two active fractions is slightly less in comparison with indomethacin. The activity of the toluene fraction at 1mg/ ear is superior to the activity of indomethacin at lower dose. The phorbol ester provides a suitable model for evaluation of both topical and systemic anti-inflammatory agents. The majority of its activities appear to involve or be dependent on arachidonic acid release and metabolism and interaction with protein kinase.

CONCLUSIONS

The ethanolic extract of leaves of *T. sulcatum* plant exhibited antioxidant activity by *in vitro* antioxidant assays. Fractionation of the total extract was done and the order of activity was found to be TSTOL > TSETOH> TSHEX> TSEA> Residue. The activity was found to be related to the total phenolic content of the extract and fractions determined by Folin Ciocalteu reagent. DPPH, β-Carotene Bleaching Assay, reducing power assays were performed. Significant dose-dependent local anti-inflammatory activity of the extract TSETOH and the hexane TSHEX as well as toluene TSTOL fractions was demonstrated. The order of activity observed was in complete agreement with that observed for the antioxidant activity. Thus the potential of *T. sulcatum* leaf extract and its fraction as natural antioxidants and anti-inflammatory agents is demonstrated.

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